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Developmental failure of the intra-articular ligaments in mice with absence of growth differentiation factor 5

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Summary

Objective: To show the phenotypic characteristics of the knee joints in brachypodism mice (bp mice), which carry a functional null mutation of the growth differentiation factor 5 (GDF5) gene, we investigated the adult and embryonic bp mice.

Method: Radiographic and macroscopic examinations of the knee joint of adult bp mice were performed. A histological examination of the knee joint of bp mice from E12.5 to E18.5 was also performed.

Results: Radiographic and macroscopic examinations of the adult bp mice showed anterior dislocation, hypoplastic condyles, and absence of the intra-articular ligaments. Safranin O staining of knee joints of the embryonic bp mice showed severe hypoplasty of the chondroepiphyses and intra-articular ligaments at E16.5. There was no difference in the number and location of 5-bromo-2′-deoxyuridine (BrdU)-positive cells between wild-type and bp mice through E12.5 to E14.5. A terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) study showed excessive cell death of mesenchymal cells of the future knee joint in bp mice at E12.5 and E13.5.

Conclusion: bp mice exhibit developmental failure of the condyles and intra-articular ligament of the knee joints.

Key words: Development, Apoptosis, Ligament formation, Knee joint, Brachypodism mice.

Introduction

Long bones begin as condensations of mesenchymal cells that arise in the early embryo, which then differentiate into prechondrogenic cells, resulting in mature chondrocytes[1]. These early skeletal structures are called a cartilaginous anlage, and will eventually become long-bone elements[1]. Simultaneously at the sites of future joints, the condensed mesenchymal cells between two separated cartilaginous anlagen remain undifferentiated[2]. These undifferentiated mesenchymal cells finally form the joint cavity, articular cartilage, and joint-associated structures such as the capsule and ligaments[2–7]. These processes require apoptosis[8–13], movement[14], and change in certain constituents of the extracellular matrix, such as hyaluronan (HA)[2,15,16] and CD44[17], the cell surface receptor of HA[18–20]. Thus, joint formation is a complex process that involves a cascade of cellular events. Growth differentiation factor 5 (GDF5), which is also known as cartilage-derived morphogenetic protein 1 belongs to a subgroup of the bone morphogenetic protein family, which is one group of signaling molecules that are known to be intimately involved in cartilage and bone formation[21,22]. GDF5 is expressed in the condensing mesenchymal cells of the limb, and later in the developing joint, during skeletal development[6,7,13,21–23]. GDF5 is essential for the formation of cartilaginous tissues during early limb development[21,22,24]. For example, GDF5 promotes the condensation of mesenchymal cells by increasing cell adhesion in the initial step of chondrogenesis[25]. In the late stage of the formation of cartilaginous tissues, when GDF5 expression is identified in the joints, GDF5 may facilitate chondrocyte proliferation in the epiphyses of the adjacent skeletal elements[25]. In addition, studies on GDF5-transgenic mice have shown that GDF5 promotes the differentiation of immature chondrocytes to mature chondrocytes[27]. Thus, it has been confirmed that GDF5 can promote chondrogenesis at any stage of development, including the condensation and proliferation of mesenchymal cells[25,26], and during chondrocyte proliferation[25], and differentiation[27].

Associations have been drawn between mutation of the human Gdf5 gene and Grebe type chondrodysplasia, Hunter-Thompson type acromesomelic chondrodysplasia and brachydactyly type C[28–30]. The most common features of these disorders are the short length of the limbs and abnormal finger joints (e.g., lack of a proximal interphalangeal joint). Mice that exhibit brachypodism (bp mice) possess the autosomal recessive gene for GDF5[22]. A frameshift mutation of the Gdf5 gene produces a translational stop at the next codon before the mature signaling portion of GDF5, and represents functional null mutations[22]. The skeletal abnormalities of bp mice are characterized by a shortened

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appendicular skeleton, including a decrease in the length of the long bones of the limbs and a reduction in the number of bones in digits 2–5, resulting in the absence of proximal interphalangeal joints in these mice[6,31,32]. Abnormal apoptosis was detected in the developing phalanges of bp mice[13], which resulted in developmental failure. Knee joints were dislocated in bp mice[31]. However, we are unaware of any other descriptions of the knee joints of bp mice. The aim of this study was to highlight the phenotypic characteristics of the knee joint in the absence of GDF5.

Materials and methods

MOUSE MAINTENANCE

The Gdf5bp-J allele occurs spontaneously in A/J strain mice (obtained from Jackson Laboratory). The allele was maintained by homozygous intercrossed breeding. For timed mating, noon on the day that the vaginal plug was observed was considered as embryonic day (E) 0.5. Approval from our Institutional Animal Care and Use Committee was obtained.

RADIOGRAPHIC AND MACROSCOPIC OBSERVATION OF THE ADULT KNEE JOINT

Eight-week-old adult bp mice ($n = 23$) were sacrificed with an intraperitoneal injection of pentobarbital, and their knee joints were dissected. Radiography was performed using Softex (SOFTEX, CMB type II, Softex Company, Tokyo, Japan), and macroscopic observation was performed with a Leica MZ-8 dissecting microscope to observe both the knee joints and joint-associated tissues.

TISSUE PROCESSING

The intra-articular tissues in bp mice were removed, embedded in paraffin, and 4 μm sections were prepared. The sections were stained with safranin O fast-green-iron hematoxylin for light microscopy.

To obtain and process fetal tissues pregnant females at E12.5–E18.5 were injected intraperitoneally with 0.01 ml/kg of 5-bromo-2′-deoxyuridine (BrdU; Roche, Mannheim, Germany). Two hours after the injection, they were killed by general anesthetic (Nembutal), and their fetuses were removed from the uterus and fixed immediately with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) overnight. They were decalcified in 10% ethylenediaminetetraacetic acid at 4°C as follows: 1 or 2 days at E14.5 and 1 week at E16.5 and E18.5. Hind limbs ($n = 205$) were dehydrated, embedded in paraffin, and 4 μm sections cut. For light microscopy, the sections were stained with safranin O fast-green-iron hematoxylin.

IN SITU HYBRIDIZATION

In situ hybridization was carried out using a method similar to that described previously[33]. Briefly, digoxigenin (DIG)-labeled, single-strand anti-sense RNA probes of GDF5, prepared with a DIG RNA labeling mix (Roche, Mannheim, Germany), were hybridized with sections containing GDF5 at 55°C for 16 h. Immunodetection of the hybridized probe was performed using an anti-DIG Fab fragment (Roche, Mannheim, Germany) overnight at 4°C. Coloring solution containing nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (an alkaline phosphatase stain; Roche, Mannheim, Germany) was applied to the slides, which were then incubated at 37°C until the signal to noise ratio was optimal.

BrdU/ANTI-BrdU IMMUNOHISTOCHEMISTRY FOR DETECTION OF PROLIFERATING CELLS

To detect cell proliferation, BrdU/Anti-BrdU immunohistochemistry was performed as described previously[34]. Briefly, after deparaffinization, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min at 15–25°C. The six sections were then digested with 0.4 mg/ml pepsin in 0.1 N HCl at 37°C for 30 min, immersed in 2 N HCl at 40°C for 1 h, and then neutralized twice using 0.1 M sodium buffer (pH 8.5). A 50-μl aliquot of anti-BrdU-Peroxidase solution was applied to each slide (Roche, Mannheim, Germany), and the sections were incubated in that solution for 1 h at 15–25°C. Immunoreactivity was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB; Wako Junyaku, Osaka, Japan).

IN SITU DETECTION OF CELL DEATH [USING THE TERMINAL DEOXYNUCLEOTIDYLTRANSFERASE-MEDIATED dUTP NICK-END LABELING (TUNEL) METHOD]

The *in situ* detection of apoptosis was performed using the ApopTag peroxidase *in situ* apoptosis detection kit (Intergen Company, New York, USA). Briefly, after deparaffination, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min at 15–25°C. The sections were pretreated with bovine testicular hyaluronidase (0.5 mg/ml in PBS at 37°C for 30 min) and then digested with proteinase K (20 μg/ml) for 10 min at room temperature. DNA was end-labeled with DIG-labeled dUTP using terminal transferase, and labeling was detected with a peroxidase-conjugated anti-DIG antibody (Intergen Company, New York, USA). DAB was used to detect the chromogen for the reaction product.

STATISTICAL ANALYSES

Statistical analyses of the data were carried out using Student's *t* test. Differences at $P < 0.05$ were considered to be significant.

Results

RADIOGRAPHIC AND MACROSCOPIC OBSERVATION OF ADULT KNEE JOINTS

Radiographic examination of adult bp mice showed that their knee joints were dislocated [Fig. 1(A,B)]. Macroscopic examination of adult bp mice showed that the femoral condyle was hypoplastic, and the patella tendon and collateral ligaments were thin. The most striking change was that the menisci and intra-articular ligaments were apparently lacking, and appeared to be included in discoid-like fibrocartilaginous tissue, which arose from the posterior part of the femoral condyle and was inserted anteriorly on the tibia [Fig. 1(D)]. The femoral articular surface made direct contact with the fibrocartilaginous tissue rather than the tibial articular surface. Histologic examination showed that this fibrocartilaginous tissue was devoid of any ligament-like structure [Fig. 1(E)]. These observations indicate that the knee joints of adult bp mice are severely dislocated and hypoplastic, and that they lack intra-articular ligaments.

SAFRANIN O STAINING OF EMBRYONIC KNEE JOINTS

Sagittal sections that appeared to be cut through the center of the embryonic knee joint were selected for examination (Fig. 2). At E12.5, condensations of mesenchymal cells appeared in embryonic skeletal elements. There

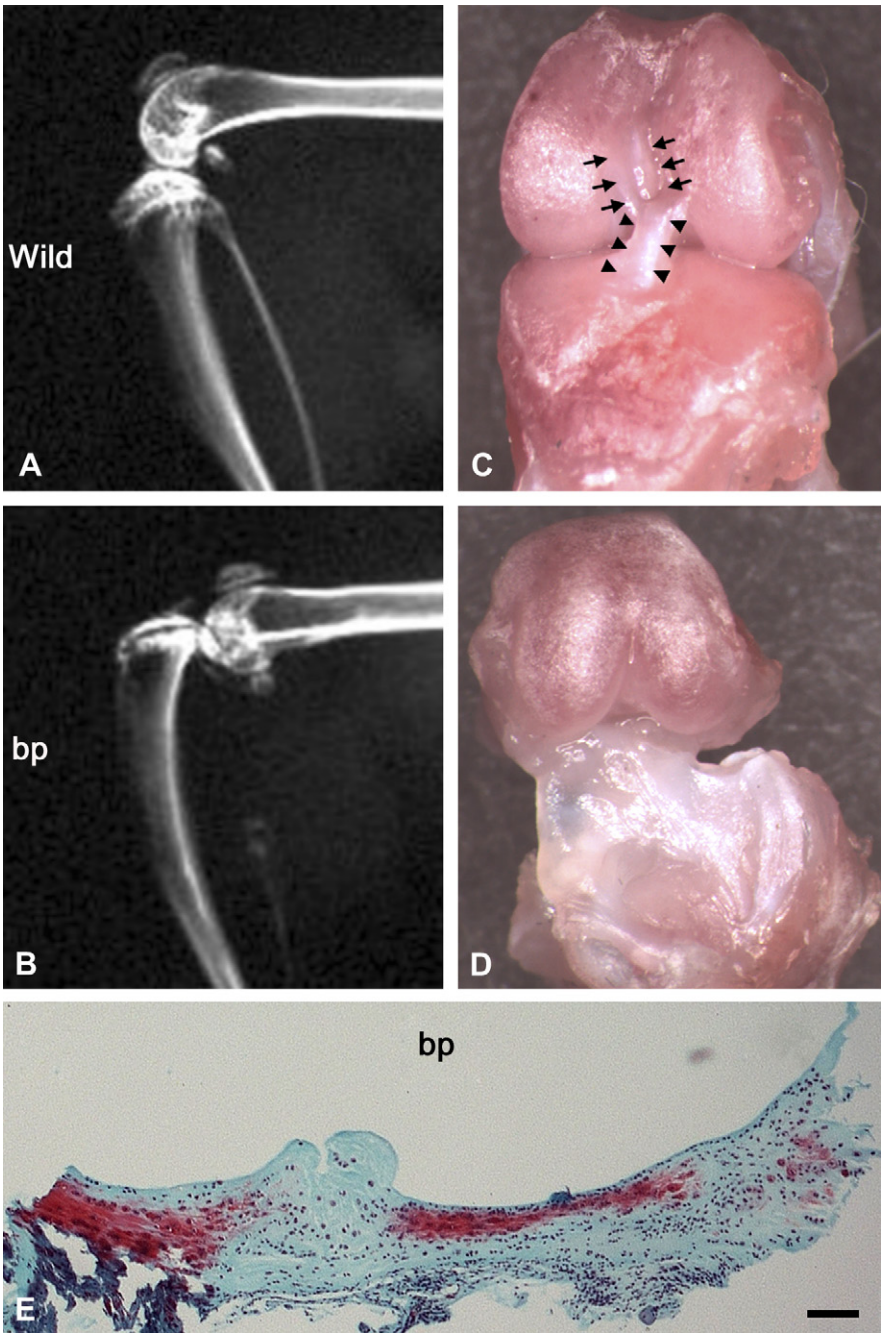

Fig. 1. Radiographic (A and B) and macroscopic (C and D) appearance of the knee joint in adult wild-type (upper panels) and bp mice (lower panels). Radiography shows dislocation of the knee joints in adult bp mice. The knee joints of wild-type mice show normal intra-articular ligaments (C, arrowheads: anterior cruciate ligament, arrows: posterior cruciate ligament), whereas bp mice have a hypoplastic femoral condyle with absence of the intra-articular ligaments (D). Safranin O staining of fibrocartilaginous tissue of knee joints in adult bp mice (coronal section). Ligament tissue was not detected (E). Bars = 100 μm.

were no differences between wild-type and bp mice in this respect [Fig. 2(A,B)]. At E13.5, two cartilaginous anlagen of the femur and tibia were stained by safranin O in both wild-type and bp mice [Fig. 2(C,D)]. A mesenchymal condensation was identified between the two cartilaginous anlagen at the site of the future knee joint. The number of condensed mesenchymal cells was counted within three 100 μm × 200 μm areas − the proximal, central, and distal parts of the future knee joints [Fig. 2(C,D)]. The central area was located at the center of the future knee joint, which was the center of the mesenchymal condensation between the two cartilaginous anlagen of the future femur and tibia. The numbers of condensed mesenchymal cells in the proximal, central, and distal areas were 213 ± 6, 207 ± 7, and 195 ± 8 in wild-type mice ($n = 6$), and 201 ± 5, 184 ± 6, and 199 ± 3 in bp mice ($n = 6$), respectively. Statistical analysis showed that there were no significant differences in these numbers between wild-type and bp mice. However

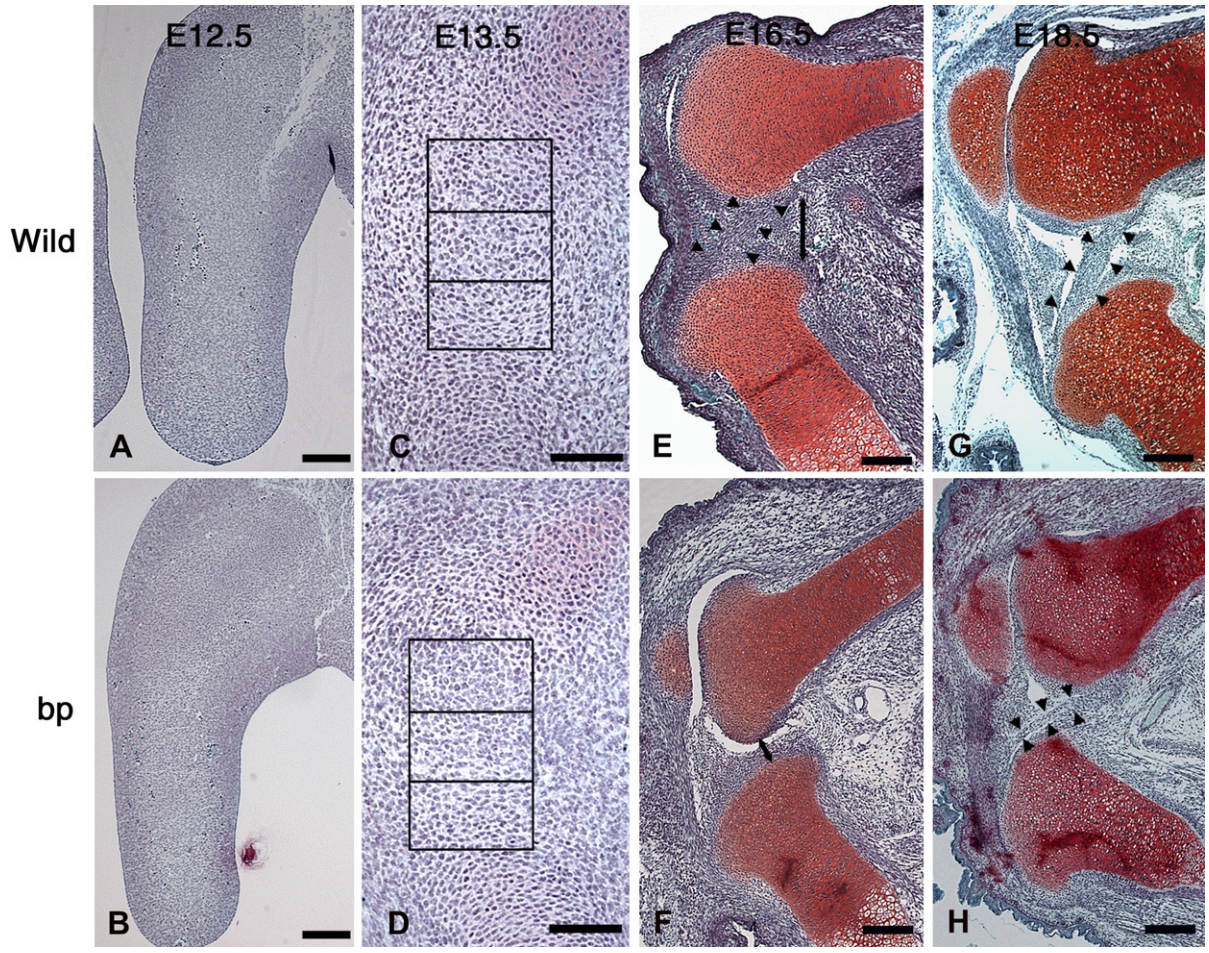

Fig. 2. Safranin O staining of knee joints in wild-type (A, C, E, and G) and bp mice (B, D, F, and H) at E12.5 (A and B), E13.5 (C and D), E16.5 (E and F), and E18.5 (G and H). At E12.5, condensations of mesenchymal cells appear in future skeletal elements in both wild-type and bp mice (A and B). At E13.5, a mesenchymal condensation of the future knee joint can be identified between two anlagen (C and D). At E16.5, while the anterior cruciate ligament (E, arrowheads) can be identified in wild-type mice, no such element is evident in bp mice (F). The femoral and tibial condyles and patella are smaller in bp mice at E16.5 (F). At E18.5, bp mice (H) exhibit anterior dislocation of the knee joint, hypoplastic condyles, and severely hypoplastic intra-articular ligaments (arrowheads). Bars = 100 μm.

the number within the central area in bp mice was less than that in wild-type mice ($P = 0.053$). At E14.5, as well as at E13.5, in both wild-type and bp mice, the two cartilaginous anlagen and mesenchymal condensation were identified (data not shown). The number of mesenchymal cells within the 100 μm × 250 μm area in the center of the future knee joint was counted. There were 422 ± 26 such cells in wild-type mice ($n = 6$), and 309 ± 14 in bp mice ($n = 6$), the number in bp mice being significantly lower ($P < 0.05$).

At E16.5 in wild-type mice, the femoral and tibial condyles, intra-articular ligaments and menisci were morphologically recognizable [Fig. 2(E)], and primary ossification had occurred in the diaphyses of the femur and tibia. In bp mice, the femoral and tibial condyles were significantly smaller, and the intra-articular ligaments could not be seen [Fig. 2(F)]. The mean size of the femoral condyle was 692 ± 35 μm (range: 625–10,811 μm) in wild-type mice, and 495 ± 28 μm (range: 430–577 μm) in bp mice. The mean size of the tibial condyle was 664 ± 26 μm (range: 589–734 μm) in wild-type mice, and 469 ± 29 μm (range: 399–560 μm) in bp mice. In addition, the distance between the cartilaginous epiphyses of the femur and tibia was significantly smaller in bp mice [Fig. 2(E,F)]. The mean distance was 283 ± 26 μm (range: 190–348 μm) in wild-type mice, and 101 ± 6 μm (range: 86–122 μm) in bp mice. At E18.5, the knee joint in wild-type mice exhibited almost complete structures such as the femoral and tibial condyles, intra-articular ligaments, menisci, and joint cavity. In contrast, the knee joints of bp mice exhibited hypoplasic femoral and tibial condyles, and severe hypoplasia or absence of the intra-articular ligaments. Membrane-like tissue, which arose from the posterior part of the femoral condyle and was inserted anteriorly on the tibia, was also observed in bp mice [Fig. 2(G,H)].

IN SITU HYBRIDIZATION OF EMBRYONIC KNEE JOINTS

At E13.5, the expression of GDF5 mRNA was detected as transverse strips at the site of the future knee joint in both types of mice [Fig. 3(C,D)]. This expression was not seen in the two cartilaginous anlagen. In bp mice, the expression of GDF5 mRNA was more intense and the area of GDF5 expression was wider [Fig. 3(C,D)]. At E14.5, expression of GDF5 mRNA was detected at the site of the future joints in both wild-type and bp mice (data not shown). There were no differences in GDF5 expression.

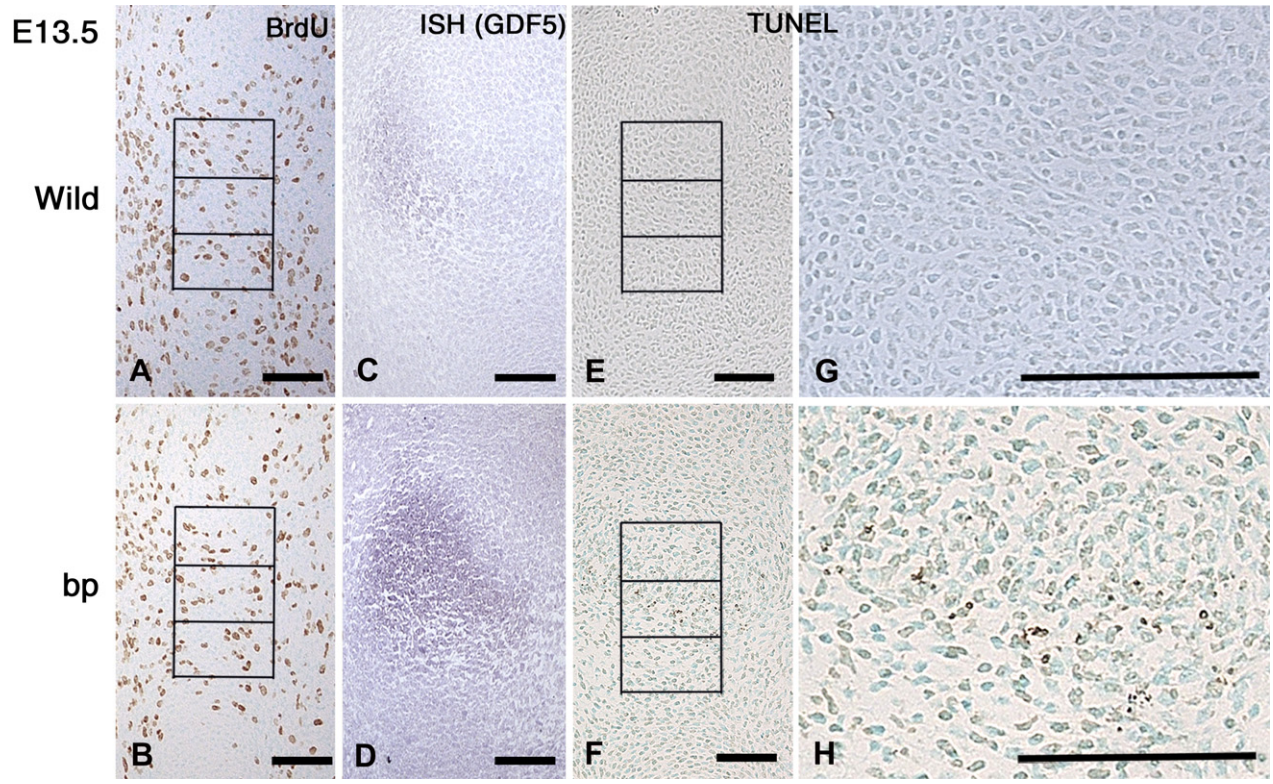

Fig. 3. Knee joints at E13.5 in wild-type (A, C, E, and G) and bp mice (B, D, F, and H). BrdU/anti-BrdU immunohistochemistry (A and B), *in situ* hybridization for GDF5 mRNA (C and D), and TUNEL staining (E, F, G, and H). G and H are higher-magnification views of E and F, respectively. In both wild-type and bp mice, BrdU-positive cells can be seen in the future knee joint, the two cartilaginous anlagen of the future femur and tibia, and the surrounding tissue (A and B). In bp mice GDF5 expression is more intense, and the area of GDF5 expression is wider (C and D). A large number of TUNEL-positive cells are evident in the future knee joint of bp mice (F and H), while only a few are observed in that of wild-type mice (E and G). Bars = 100 μm.

CELL PROLIFERATION IN EMBRYONIC KNEE JOINTS

The number of BrdU-positive cells was measured at E12.5–E14.5 to clarify the status of cell proliferation. At E12.5, BrdU-positive cells in both wild-type and bp mice were seen in condensations of mesenchymal cells and in the surrounding tissue (data not shown). At E13.5 and E14.5, in both wild-type and bp mice, BrdU-positive cells were seen in future knee joint tissue, the two cartilaginous anlagen of the future femur and tibia, and the surrounding tissue [Fig. 3(A,B)]. The location of these cells in wild-type and bp mice appeared to be similar. From E12.5 to E14.5, the number of BrdU-positive cells in the three 100 μm × 200 μm areas, previously defined, was counted [Fig. 3(A,B)]. In each area there was no difference in the number of BrdU-positive cells between wild-type and bp mice at any stage (Table I).

CELL DEATH IN EMBRYONIC KNEE JOINTS

At E12.5, TUNEL-positive cells were observed in condensations of mesenchymal cells in both wild-type and bp mice (data not shown). The number of TUNEL-positive cells was

Table I
Cell proliferation in future distal femur, knee joint, and proximal tibia at E12.5, E13.5, and E14.5

		Number of BrdU-positive cells					
		E12.5		E13.5		E14.5	
		Wild ($n=3$)	bp ($n=6$)	Wild ($n=8$)	bp ($n=6$)	Wild ($n=9$)	bp ($n=6$)
Area	Proximal	28.3 ± 9.9	28.6 ± 1.4	21.8 ± 1.9	28.0 ± 3.0	14.6 ± 1.3	13.2 ± 1.8
	Central	31.0 ± 3.7	24.1 ± 2.5	16.8 ± 2.2	17.3 ± 2.4	21.7 ± 1.4	19.5 ± 1.3
	Distal	32.6 ± 4.0	23.6 ± 2.5	28.4 ± 2.4	28.8 ± 3.8	22.4 ± 1.0	21.8 ± 2.3

At E12.5, E13.5, and E14.5, number of BrdU-positive cells were individually measured within three areas (100 μm x 200 μm); proximal, central, and distal. At E12.5, central area is placed on the one which is considered to be center of mesenchymal condensation. At E13.5 and E14.5, central area is placed on the one which is considered to be mesenchymal condensation between two cartilaginous anlagen of future femur and tibia. Values are the mean ± S.E.M. There are no significant difference between wild-type and bp mice (Student's *t* test).

Table II
Apoptosis in future distal femur, knee joint, and proximal tibia at E12.5, E13.5, and E14.5

		Number of TUNEL-positive cells					
		E12.5		E13.5		E14.5	
		Wild ($n=5$)	bp ($n=4$)	Wild ($n=13$)	bp ($n=5$)	Wild ($n=14$)	bp ($n=12$)
Area	Proximal	2.0 ± 1.3	13.5 ± 3.9*	0.1 ± 0.1	1.5 ± 0.9*	0.1 ± 0.0	0.2 ± 0.1
	Central	2.2 ± 1.0	12.7 ± 4.9*	0.2 ± 0.1	8.0 ± 1.9**	0.0 ± 0.0	0.2 ± 0.1
	Distal	2.6 ± 1.4	10.7 ± 2.4*	0.3 ± 0.2	3.0 ± 1.6*	0.0 ± 0.0	0.0 ± 0.0

At E12.5, E13.5, and 14.5, number of TUNEL-positive cells were measured, just as in Table I. Values are the mean $\pm$ S.E.M. P values are calculated using Student's t test (*$P < 0.05$, **$P < 0.0001$). At both E12.5 and E13.5, in each areas, the number of TUNEL-positive cells in bp mice is significantly greater than in wild-type mice. At E13.5, there is excessive apoptosis especially in the future knee joint area between two cartilaginous anlagen in bp mice.

measured within the three defined 100 μm $\times$ 200 μm areas. In each area, the number of TUNEL-positive cells in bp mice was significantly greater than that in wild-type mice at E12.5 (Table II). At E13.5 there was excessive apoptosis at the site of the future knee joint in bp mice [Fig. 3(E–H)]. After E14.5, quite a few TUNEL-positive cells were observed in both wild-type and bp mice (Table II).

Discussion

In the present study, radiographic and macroscopic examinations of adult bp mice showed anterior dislocation, hypoplastic condyles, and absence of the intra-articular ligaments. Histological examination of embryonic bp mice showed severe hypoplasia of the condyles and intra-articular ligaments. These findings suggest developmental failure of the condyles and intra-articular ligament of the knee joints in bp mice. There was no difference in the number and location of BrdU-positive cells between wild-type and bp mice at E12.5, E13.5, and E14.5. However, the number of TUNEL-positive cells in bp mice was significantly greater at the site of the future knee joint at E12.5 and E13.5. The condensation of mesenchymal cells at the site of the future knee joints in bp mice was decreased at E14.5. At E16.5, the distance between the cartilaginous epiphyses of the femur and tibia was significantly smaller. These results suggest that the excessive cell death, including apoptosis and necrosis, in the future knee joint of bp mice results in a decrease of mesenchymal cells. In the present study, overexpression of GDF5 mRNA was observed in the mesenchymal condensation between the two cartilaginous anlagen of the future femur and tibia at E13.5. Excessive cell death of mesenchymal cells was observed in the area where overexpression was seen. Further study is required to clarify the relationship between cell death of mesenchymal cells and the absence of GDF5.

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